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<p>(54) Title: A NOVEL HAEMOPOIETIN RECEPTOR AND GENETIC SEQUENCES ENCODING SAME  (57) Abstract  The present invention is directed to a novel haemopoietin receptor or a derivative thereof and to genetic sequences encoding same. The receptor molecule and its derivatives and the genetic sequences encoding same of the present invention are useful in the development of a wide range of agonists, antagonists, therapeutics and diagnostic reagents based on ligand interaction with its receptor. The present invention particularly relates to a <u>receptor for leptin</u>.  <i>Marked at 19</i></p>		

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## **A NOVEL HAEMOPOIETIN RECEPTOR AND GENETIC SEQUENCES ENCODING SAME**

5 The present invention is directed to a novel haemopoietin receptor or a derivative thereof and to genetic sequences encoding same. The receptor molecule and its derivatives and the genetic sequences encoding same of the present invention are useful in the development of a wide range of agonists, antagonists, therapeutics and diagnostic reagents based on ligand interaction with its receptor. The present invention particularly relates to a receptor for leptin.

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Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID NOs.) for the nucleotide and amino acid sequences referred to in the specification are defined following the bibliography.

15

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

20

The preferred haemopoietin receptor of the present invention is referred to herein as "NR2". The NR2 receptor interacts with leptin and is referred to as a "leptin receptor". The terms "haemopoietin receptor", "NR2" and "leptin receptor" are used interchangeably throughout the subject specification. The species from which a particular NR2 is desired is given in single letter abbreviation in lower case before NR2. For example, murine NR2 is "mNR2" and human NR2 is "hNR2". A recombinant form may have the prefix "r".

The rapidly increasing sophistication of recombinant DNA techniques is greatly facilitating research into the medical and allied health fields. Cytokine research is of particular importance, especially as these molecules regulate the proliferation, differentiation and function of a wide

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variety of cells. Administration of recombinant cytokines or regulating cytokine function and/or synthesis is becoming increasingly the focus of medical research into the treatment of a range of disease conditions.

- 5 Despite the discovery of a range of cytokines and other secreted regulators of cell function, comparatively few cytokines are directly used or targeted in therapeutic regimens. One reason for this is the pleiotropic nature of many cytokines. For example, interleukin (IL)-11 is a functionally pleiotropic molecule (1,2), initially characterized by its ability to stimulate proliferation of the IL-6-dependent plasmacytoma cell line, T11 65 (3). Other biological
- 10 actions of IL-11 include induction of multipotential haemopoietin progenitor cell proliferation (4,5,6), enhancement of megakaryocyte and platelet formation (7,8,9,10), stimulation of acute phase protein synthesis (11) and inhibition of adipocyte lipoprotein lipase activity (12, 13). The diverse and pleiotropic function of IL-11 and other haemopoietin cytokines makes these molecules an important group to study, especially at the level of interaction of the cytokines
- 15 with their receptors. Manipulation and control of cytokine receptors and of cytokine-receptor interaction is potentially very important in many therapeutic situations, especially where the target cytokine is functionally pleiotropic and it is desired to block certain functions of a target cytokine but not all functions.
- 20 Another important aspect of cytokine receptors is in the search for new cytokines. In this regard, the inventors have used a procedure for cloning haemopoietin receptors without prior knowledge of their ligands. Identification of receptors then provides a screening procedure for potentially new cytokines and for previously characterised cytokines. In addition, identification of new haemopoietin receptors allows for selective blocking of pleiotropic cytokine function.
- 25 In accordance with the present invention, the inventors identified a novel haemopoietin receptor which interacts with leptin, a hormone which regulates adipose tissue mass.

Accordingly, one aspect of the present invention is directed to an isolated nucleic acid molecule

30 comprising a sequence of nucleotides encoding or complementary to a sequence encoding a

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haemopoietin receptor or a derivative thereof wherein said sequence of nucleotides or a complementary form thereof is capable of hybridising under medium stringent conditions to the oligonucleotide:

5'-(A/G)CTCCA(A/G)TC(A/G)CTCCA-3' [SEQ ID NO:1].

5

In a preferred embodiment, the nucleic acid molecule comprises a nucleotide sequence or a complementary form thereof which hybridises under medium stringent conditions to the oligonucleotides:

5'-(A/G)CTCCA(A/G)TC(A/G)CTCCA-3' [SEQ ID NO:1]

10 5'-ACTAGCAGGGATGTAGCTGAG-3' [SEQ ID NO:4]

5'-CTGCTCCTATGATACCT-3' [SEQ ID NO:6]

5'-CCTCTTCCATCTTATTGCTTGG-3' [SEQ ID NO:7]

5'-ATCGGTCGTGACATACAAGG-3' [SEQ ID NO:8].

15 In an even more preferred embodiment, the nucleic acid molecule comprises a nucleotide sequence or a complementary form thereof which hybridises under medium stringent conditions to one or more of the following oligonucleotides:

5'-(A/G)CTCCA(A/G)TC(A/G)CTCCA-3' [SEQ ID NO:1].

5'-ACTAGCAGGGATGTAGCTGAG-3' [SEQ ID NO:4]

20 5'-CTCAGCTACATCCCTGCTAGT-3' [SEQ ID NO:5]

5'-CTGCTCCTATGATACCT-3' [SEQ ID NO:6]

5'-CCTCTTCCATCTTATTGCTTGG-3' [SEQ ID NO:7]

5'-ATCGGTCGTGACATACAAGG-3' [SEQ ID NO:8]

5'-AGCTAAGCTTTCTAGATATCCAATTACTCCTTGGAGA-3' [SEQ ID NO:9]

25 5'-AGCTTCTAGATCAATCACTCTGGTGTTTTTCAAT-3' [SEQ ID NO:10]

5'-AGCTTCTAGATCAAACCTTTTATATCCATGACAAC-3' [SEQ ID NO:11].

In a still more preferred embodiment, the nucleic acid molecule comprises a nucleotide sequence or complementary form thereof which is capable of hybridising separately under  
30 medium stringent conditions to each of oligonucleotide SEQ ID NO:1 and SEQ ID NO:4 to

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SEQ ID NO:11.

In a most preferred embodiment, the present invention provides a nucleic acid molecule comprising a sequence of nucleotides or a complementary form thereof substantially as set forth  
5 in Figure 2 [SEQ ID NO:12] or a sequence of nucleotides capable of hybridising to all or part thereof under medium stringent conditions.

Accordingly, a preferred embodiment of the present invention is also directed to a nucleic acid molecule encoding a haemopoietin receptor or a derivative thereof and comprising a nucleotide  
10 sequence as set forth in SEQ ID NO:12 or is capable of hybridising to all or part thereof under medium stringent conditions.

The haemopoietin receptor of the present invention is referred to herein as "NR2". In accordance with the present invention, NR2 is capable of interacting with leptin and, hence, is  
15 also referred to as a "leptin receptor".

The term "derivative" includes any or all parts, fragments, portions, homologues or analogues to the nucleotide sequence set forth in SEQ ID NO:12 as well as hybrid molecules between the NR2 receptor and other receptors or other molecules. Derivatives include single or multiple  
20 nucleotide substitutions, deletions and/or additions to the nucleotide sequence set forth in SEQ ID NO:12.

Another aspect of the present invention contemplates a recombinant haemopoietin receptor encoded by the nucleic acid molecules as hereinbefore described.

25

According to one aspect of this embodiment, there is provided recombinant haemopoietin receptor encoded by a nucleic acid molecule which comprises a nucleotide sequence or a complementary form thereof which is capable of hybridising to SEQ ID NO:1 under medium stringent conditions.

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In a preferred embodiment, the recombinant haemopoietin receptor is encoded by a nucleic acid molecule which comprises a nucleotide sequence or a complementary form thereof which is capable of hybridising to SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8 under medium stringent conditions.

5

In an even more preferred embodiment, the recombinant haemopoietin receptor is encoded by a nucleic acid molecule which comprises a nucleotide sequence or complementary form thereof which hybridises under medium stringency conditions to one or more of SEQ ID NO:1 and SEQ ID NO:4 to SEQ ID NO:11.

10

In still an even more preferred embodiment, the recombinant haemopoietin receptor is encoded by a nucleic acid molecule which comprises a nucleotide sequence or a complementary form thereof which hybridises under medium stringent conditions to each of oligonucleotides SEQ ID NO:1 and SEQ ID NO:4 to SEQ ID NO:11.

15

In a most preferred embodiment, the present invention is directed to a recombinant NR2 encoded by a nucleic acid molecule comprising a nucleotide sequence or complementary form thereof substantially as set forth in SEQ ID NO:12 or a sequence capable of hybridising to all or part thereof under medium stringent conditions.

20

According to this latter aspect of the present invention, there is provided a recombinant NR2 having an amino acid sequence substantially as set forth in Figure 2 [SEQ ID NO:13] or having at least about 60% similarity to all or part thereof, more preferably at least about 70%, still more preferably at least about 80% and still more preferably at least about 90-95% or above  
25 (e.g. 96%, 97%, 98% or greater than or equal to 99%) similarly to all or part of the amino acid sequence set forth in SEQ ID NO:13.

The recombinant NR2 or a genetic sequence encoding same is preferably in isolated form meaning that a composition of matter comprises at least about 10%, more preferably at least  
30 about 20%, still more preferably at least about 30-40%, even more preferably at least about 50-

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60%, still even more preferably at least about 70-80% or greater (e.g. 85%, 90% or 95%) of the recombinant receptor or genetic sequence encoding same relative to other components in the composition as determined by, for example, molecular weight, activity, nucleic acid content or composition or other convenient means.

5

Reference herein to "recombinant haemopoietin receptor", "NR2" or "leptin receptor" includes reference to derivatives thereof such as parts, fragments, portions, homologues, hybrids or analogues thereof. The derivatives may be functional or not or may be non-functional but immunologically interactive with antibodies to all or part of the receptor. Derivatives of the receptor also cover agonists or antagonists of receptor-ligand interaction. Function is conveniently defined by an ability of NR2 to interact with leptin or for soluble NR2 to compete with leptin-induced activities of certain cells.

For the purposes of defining the level of stringency, reference can conveniently be made to Sambrook *et al* (14) which is herein incorporated by reference where the washing steps disclosed at pages 952-957 are considered high stringency. A low stringency is defined herein as being in 4-6X SSC/0.1-0.5% w/v SDS at 37-45°C for 2-3 hours. Depending on the source and concentration of nucleic acid involved in the hybridisation, alternative conditions of stringency may be employed such as medium stringent conditions which are considered herein to be 1-4X SSC/0.25-0.5% w/v SDS at  $\geq 45^{\circ}\text{C}$  for 2-3 hours or high stringent conditions considered herein to be 0.1-1X SSC/0.1% w/v SDS at  $\geq 60^{\circ}\text{C}$  for 1-3 hours.

The nucleic acid molecule is preferably derivable from the human genome but genomes and nucleotide sequences from non-human animals are also encompassed by the present invention. Non-human animals contemplated by the present invention include livestock animals (e.g. sheep, cows, pigs, goats, horses, donkeys), laboratory test animals (e.g. mice, rats, guinea pigs, hamsters, rabbits), domestic companion animals (e.g. dogs, cats), birds (e.g. chickens, geese, ducks and other poultry birds, game birds, emus, ostriches) and captive wild or tamed animals (e.g. foxes, kangaroos, dingoes).

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Preferred human genetic sequences encoding NR2 include sequences from cells of bone marrow, brain, liver, kidney, heart, testis, stomach, lymph nodes, colon, spleen and ovary, neonatal tissue, embryonic tissue, cancer or tumour-derived tissues.

5 The nucleic acid molecule of the present invention may be single or double stranded, linear or closed circle DNA (e.g. genomic DNA), cDNA or mRNA or combinations thereof such as in the form of DNA:RNA hybrids. The nucleic acid molecule may also include a vector such as an expression vector component to facilitate expression of the haemopoietin receptor or its components or parts.

10

As stated above, the present invention further contemplates a range of derivatives of NR2. Derivatives include fragments, parts, portions, mutants, homologues and analogues of the NR2 polypeptide and corresponding genetic sequence. Derivatives also include single or multiple amino acid substitutions, deletions and/or additions to NR2 or single or multiple nucleotide  
15 substitutions, deletions and/or additions to the genetic sequence encoding NR2. "Additions" to amino acid sequences or nucleotide sequences include fusions with other peptides, polypeptides or proteins or fusions to nucleotide sequences. Reference herein to "NR2" includes reference to all derivatives thereof including functional derivatives or "NR2" immunologically interactive derivatives.

20

Analogues of NR2 contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogues.

25

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with  $\text{NaBH}_4$ ; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups  
30 with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic



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anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with  $\text{NaBH}_4$ .

The guanidine group of arginine residues may be modified by the formation of heterocyclic  
5 condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

10 Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol  
15 and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form  
20 a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

25 Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table

30 1.

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Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having  $(CH_2)_n$  spacer groups with  $n=1$  to  $n=6$ , glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group  
5 specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of  $C_\alpha$  and  $N_\alpha$ -methylamino acids, introduction of double bonds between  $C_\alpha$  and  $C_\beta$  atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a  
10 side chain and the N or C terminus.

These types of modifications may be important to stabilise NR2 if administered to an individual or for use as a diagnostic reagent.

15 The present invention further contemplates chemical analogues of NR2 capable of acting as antagonists or agonists of NR2 or which can act as functional analogues of NR2. Chemical analogues may not necessarily be derived from NR2 but may share certain conformational similarities. Alternatively, chemical analogues may be specifically designed to mimic certain physiochemical properties of NR2. Chemical analogues may be chemically synthesised or may  
20 be detected following, for example, natural product screening.

The identification of NR2 permits the generation of a range of therapeutic molecules capable of modulating expression of NR2 or modulating the activity of NR2. Modulators contemplated by the present invention includes agonists and antagonists of NR2 expression. Antagonists of  
25 NR2 expression include antisense molecules, ribozymes and co-suppression molecules. Agonists include molecules which increase promoter ability or interfere with negative regulatory mechanisms. Agonists of NR2 include molecules which overcome any negative regulatory mechanism. Antagonists of NR2 include antibodies and inhibitor peptide fragments.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 $\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
10 aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine		L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
15 D-alanine	Dal	Chexa L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
20 D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
25 D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
30 D-threonine	Dthr	L-N-methylethylglycine	Nmetg
		L-N-methyl-t-butylglycine	Nmtbug
		L-norleucine	Nle

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	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
	D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgabu
	D- $\alpha$ -methylalanine	Dmala	$\alpha$ -methylcyclohexylalanine	Mchexa
5	D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
	D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
	D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
	D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norm
	D- $\alpha$ -methylisoleucine	Dmile	N-amino- $\alpha$ -methylbutyrate	Nmaabu
	D- $\alpha$ -methyllleucine	Dmleu	$\alpha$ -naphthylalanine	Anap
	D- $\alpha$ -methylllysine	Dmlys	N-benzylglycine	Nphe
	D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15	D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- $\alpha$ -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- $\alpha$ -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D- $\alpha$ -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
30	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser

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	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl)glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
5	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
10	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl- $\alpha$ -naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
15	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
	L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylasparagine	Masn
	L- $\alpha$ -methylaspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
	L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
20	L- $\alpha$ -methylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
	L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomophenylalanine	Mhphe
	L- $\alpha$ -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L- $\alpha$ -methylleucine	Mleu	L- $\alpha$ -methyllysine	Mlys
	L- $\alpha$ -methylmethionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
25	L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
	L- $\alpha$ -methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
	L- $\alpha$ -methylserine	Mser	L- $\alpha$ -methylthreonine	Mthr
	L- $\alpha$ -methyltryptophan	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr

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L- $\alpha$ -methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl)	Nnbhm	N-(N-(3,3-diphenylpropyl)	Nnbhe
carbamylmethyl)glycine		carbamylmethyl)glycine	
1-carboxy-1-(2,2-diphenyl-	Nmbc		
5 ethylamino)cyclopropane			

Other derivatives contemplated by the present invention include a range of glycosylation variants from a completely unglycosylated molecule to a modified glycosylated molecule.

10 Altered glycosylation patterns may result from expression of recombinant molecules in different host cells.

Another embodiment of the present invention contemplates a method for modulating expression of NR2 in a human, said method comprising contacting the NR2 gene encoding

15 NR2 with an effective amount of a modulator of NR2 expression for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of NR2. For example, a nucleic acid molecule encoding NR2 or a derivative thereof may be introduced into a cell to enhance NR2 related activities of that cell. Conversely, NR2 antisense sequences (or sense sequences for co-suppression) such as oligonucleotides may be

20 introduced to decrease NR2-related activities of any cell expressing the endogenous NR2 gene. Ribozymes may also be used.

Another aspect of the present invention contemplates a method of modulating activity of NR2 in a human, said method comprising administering to said mammal a modulating effective

25 amount of a molecule for a time and under conditions sufficient to increase or decrease NR2 activity. The molecule may be a proteinaceous molecule or a chemical entity and may also be a derivative of NR2 or its receptor or a chemical analogue or truncation mutant of NR2 or its receptor.

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Accordingly, the present invention contemplates a pharmaceutical composition comprising NR2 or a derivative thereof or a modulator of NR2 expression or NR2 activity and one or more pharmaceutically acceptable carriers and/or diluents. These components are referred to as the "active ingredients".

5

In this regard there is provided a pharmaceutical composition comprising a recombinant haemopoietin receptor as hereinbefore described or a ligand (e.g. leptin) binding portion thereof and one or more pharmaceutically acceptable carriers and/or diluents.

- 10 In another embodiment, there is provided a pharmaceutical composition comprising a ligand (e.g. leptin) to the recombinant haemopoietin receptor as hereinbefore described and one or more pharmaceutically acceptable carriers and/or diluents.

- Still a further aspect of the present invention contemplates a method of treatment of an animal comprising administering to said animal a treatment effective amount of a recombinant haemopoietin receptor as hereinbefore described or a ligand binding portion thereof or a ligand (e.g. leptin) to said haemopoietic receptor for a time and under conditions sufficient for said treatment to be substantially effected or the conditions to be substantially ameliorated.

20

- The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as licithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The preventions of the
- 25  
30

- 15 -

action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the  
5 compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by  
10 incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously  
15 sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be  
20 incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may  
25 conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 ug and 2000 mg of active compound.



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The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such a sucrose, lactose or  
5 saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound,  
10 sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

15

The present invention also extends to forms suitable for topical application such as creams, lotions and gels.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion  
20 media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

25

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated, each unit containing a predetermined quantity of active material calculated to produce the  
30 desired therapeutic effect in association with the required pharmaceutical carrier. The

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specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which

5   bodily health is impaired.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active

10   compound in amounts ranging from 0.5  $\mu$ g to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5  $\mu$ g to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

15

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating NR2 expression or NR2 activity. The vector may, for example, be a viral vector.

20

Still another aspect of the present invention is directed to antibodies to NR2 and its derivatives or its ligands (e.g. leptin). Such antibodies may be monoclonal or polyclonal and may be selected from naturally occurring antibodies to NR2 or may be specifically raised to NR2 or derivatives thereof. In the case of the latter, NR2 or its derivatives may first need to be

25   associated with a carrier molecule. The antibodies and/or recombinant NR2 or its derivatives of the present invention are particularly useful as therapeutic or diagnostic agents.

For example, NR2 and its derivatives can be used to screen for naturally occurring antibodies to NR2. These may occur, for example in some autoimmune diseases. Alternatively, specific

30   antibodies can be used to screen for NR2. Techniques for such assays are well known in the

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art and include, for example, sandwich assays and ELISA. Knowledge of NR2 levels may be important for diagnosis of certain cancers or a predisposition to cancers or for monitoring certain therapeutic protocols.

- 5 Antibodies to NR2 of the present invention may be monoclonal or polyclonal. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy  
10 and may also be used as a diagnostic tool for assessing the receptor or receptor-ligand interaction or monitoring the program of a therapeutic regimen.

For example, specific antibodies can be used to screen for NR2 proteins. The latter would be important, for example, as a means for screening for levels of NR2 in a cell extract or other  
15 biological fluid or purifying NR2 made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays and ELISA.

It is within the scope of this invention to include any second antibodies (monoclonal,  
20 polyclonal or fragments of antibodies or synthetic antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of NR2.

25

Both polyclonal and monoclonal antibodies are obtainable by immunization with the enzyme or protein and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of NR2, or  
30 antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any

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of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

- 5 The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art.

10

Another aspect of the present invention contemplates a method for detecting NR2 in a biological sample from a subject said method comprising contacting said biological sample with an antibody specific for NR2 or its derivatives or homologues for a time and under conditions sufficient for an antibody-NR2 complex to form, and then detecting said complex.

15

- The presence of NR2 may be accomplished in a number of ways such as by Western blotting and ELISA procedures. A wide range of immunoassay techniques are available as can be seen by reference to US Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These, of course, includes both single-site and two-site or "sandwich" assays of the non-competitive types, as  
20 well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

- Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist,  
25 and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a  
30 detectable signal is then added and incubated, allowing time sufficient for the formation of

- 20 -

another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain NR2 including cell extract, tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising biological fluid, cell extract, bone marrow or lymph, tissue extract (e.g. from kidney, liver, spleen, etc), fermentation fluid and supernatant fluid such as from a cell culture and cell conditioned medium.

15 In the typical forward sandwich assay, a first antibody having specificity for the NR2 or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes) and under suitable conditions (e.g. 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

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An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody.

- 5 Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its  
10 chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody,  
15 generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the  
20 corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate  
25 substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

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Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention also contemplates genetic assays such as involving PCR analysis to detect NR2 gene or its derivatives. Alternative methods or methods used in conjunction include direct nucleotide sequencing or mutation scanning such as single stranded conformation polymorphisms analysis (SSCP) as specific oligonucleotide hybridisation, as methods such as direct protein truncation tests. Such genetic tests may be important, for example, in genetic screening of animals (e.g. humans) for non-expression or substantial absence of expression or expression of mutant forms of NR2 leading to conditions such as obesity or other effects of leptin-receptor interaction.

The nucleic acid molecules of the present invention may be DNA or RNA. When the nucleic acid molecule is in DNA form, it may be genomic DNA or cDNA. RNA forms of the nucleic acid molecules of the present invention are generally mRNA.

25

Although the nucleic acid molecules of the present invention are generally in isolated form, they may be integrated into or ligated to or otherwise fused or associated with other genetic molecules such as vector molecules and in particular expression vector molecules. Vectors and expression vectors are generally capable of replication and, if applicable, expression in one or both of a prokaryotic cell or a eukaryotic cell. Preferably, prokaryotic cells include *E.*

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*coli*, *Bacillus sp* and *Pseudomonas sp*. Preferred eukaryotic cells include yeast, fungal, mammalian and insect cells.

Accordingly, another aspect of the present invention contemplates a genetic construct  
5 comprising a vector portion and a mammalian and more particularly a human NR2 gene  
portion, which NR2 gene portion is capable of encoding an NR2 polypeptide or a functional  
or immunologically interactive derivative thereof.

Preferably, the NR2 gene portion of the genetic construct is operably linked to a promoter on  
10 the vector such that said promoter is capable of directing expression of said NR2 gene portion  
in an appropriate cell.

In addition, the NR2 gene portion of the genetic construct may comprise all or part of the gene  
fused to another genetic sequence such as a nucleotide sequence encoding glutathione-S-  
15 transferase or part thereof or a cytokine or another haemopoietic receptor. Hybrid receptor  
molecules are particularly useful in the development of multi functional therapeutic and  
diagnostic agents.

The present invention extends to such genetic constructs and to prokaryotic or eukaryotic cells  
20 comprising same.

The present invention also extends to any or all derivatives of NR2 including mutants, part,  
fragments, portions, homologues and analogues or their encoding genetic sequence including  
single or multiple nucleotide or amino acid substitutions, additions and/or deletions to the  
25 naturally occurring nucleotide or amino acid sequence.

The NR2 and its genetic sequence of the present invention will be useful in the generation of  
a range of therapeutic and diagnostic reagents and will be especially useful in the detection of  
a corresponding ligand. For example, recombinant NR2 may be bound or fused to a reporter  
30 molecule capable of producing an identifiable signal, contacted with a biological sample



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putatively containing a ligand and screening for binding of the labelled NR2 to the ligand. Alternatively, labelled NR2 may be used to screen expression libraries of putative ligand genes or functional parts thereof.

- 5 In another embodiment, the NR2 is first immobilised. According to this embodiment, there is provided a method comprising contacting a biological sample containing a putative ligand with said haemopoietic receptor or a ligand binding portion thereof immobilised to a solid support for a time and under conditions sufficient for a complex to form between said receptor and said ligand if said ligand is present in said biological sample, eluting bound  
10 ligand and isolating same.

Soluble NR2 polypeptides are also contemplated to be useful in the treatment of disease, injury or abnormality in the nervous system, e.g. in relation to central or peripheral nervous system to treat Cerebral Palsy, trauma induced paralysis, vascular ischaemia associated with stroke,  
15 neuronal tumours, motoneurone disease, Parkinson's disease, Huntington's disease, Alzheimer's disease, Multiple Sclerosis, peripheral neuropathies associated with diabetes, heavy metal or alcohol toxicity, renal failure and infectious diseases such as herpes, rubella, measles, chicken pox, HIV or HTLV-1. The NR2 polypeptides may also be important for regulating cytokine activity such as leptin activity, modulating haemopoiesis and/or regulating  
20 or modulating adipose tissue.

As stated above, the NR2 or its ligand of the present invention or their functional derivatives may be provided in a pharmaceutical composition together with one or more pharmaceutically acceptable carriers and/or diluents. In addition, the present invention contemplates a method  
25 of treatment comprising the administration of an effective amount of NR2 of the present invention. The present invention also extends to antagonists and agonists of NR2 and/or its ligand and their use in therapeutic compositions and methodologies.

A further aspect of the present invention contemplates the use of NR2 or its functional  
30 derivatives in the manufacture of a medicament for the treatment of NR2 mediated conditions

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defective or deficient.

The present invention is further described with reference to the following non-limiting Figures and/or Examples.

5

In the Figures:

**Figure 1** is a schematic representation showing size of NR2 cDNA clones isolated and schematic structure of the predicted NR2 protein.

10

**Figure 2** is a representation of the nucleotide sequence and corresponding amino acid sequence of the haemopoietin receptor.

**Figure 3** is a representation of a FACS analysis of NR2 expression by BA/F<sub>3</sub> cells.

15

**Figure 4** is a photographic representation of a silver-stained gel of NR2 expression by BA/F<sub>3</sub> cells.

**Figure 5** is a graphical representation showing specific binding of <sup>125</sup>I human leptin to Ba/F<sub>3</sub> cells stably transfected to express hNR2 on their cell surface.

20

- (a) Saturation binding curve for <sup>125</sup>I h leptin binding to Ba/F<sub>3</sub>/hNR2 cells at 23° C.
- (b) Scatchard transformation of the data in (a). The slope of the curve indicates an equilibrium dissociation constant ( $K_D$ ) of 120 pM.

**Figure 6** is a graphical representation showing specific binding of <sup>125</sup>I human leptin to COS-7 cells transiently transfected to express hNR2 on their cell surface (a) or to purified soluble human NR2 (b). Saturation binding curves at 23° C are shown.

**Figure 7** is a photographic representation showing cross species conservation of the NR2 gene. Southern blot of genomic DNA probed with a specific cDNA probe for NR2.

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**Figure 8** is a diagrammatic representation of the NR2 locus. A map of the NR2 locus, showing positioning of the clones isolated from genomic libraries. The results of the restriction enzyme mapping using NcoI and the positioning of the exons on these fragments are also shown.

5

**Figure 9** is a photographic representation showing expression of leptin receptor (NR2) in murine tissues.

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The following single and three letter abbreviations for amino acid residues are used in the specification:

5 Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
10 Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
15 Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
20 Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
25 Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

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**TABLE 2**  
**SEQUENCE OF OLIGONUCLEOTIDES**

5	OLIGONUCLEOTIDE	SEQUENCE	SEQ ID NOs
	HYB2	5'-(A/G)CTCCA(A/G)TC(A/G)CTCCA-3'	1
10	T3	5'-TAATACGACTCACTATAGGGAGA-3'	2
	T7	5'-ATTAACCCTCACTAAAGGGA-3'	3
	721	5'-ACTAGCAGGGATGTAGCTGAG-3'	4
	722	5'-CTCAGCTACATCCCTGCTAGT-3'	5
	761	5'-CTGCTCCTATGATACCT-3'	6
15	875	5'-CCTCTTCCATCTTATTGCTTGG-3'	7
	939	5'-ATCGGTCGTGACATAACAAGG-3'	8
	1056	5'AGCTAAGCTTTCTAGATATCCAATTACTCCTTGGAGA-3'	9
	1092	5'-AGCTTCTAGATCAATCACTCTGGTGTTCAT-3'	10
	1094	5'-AGCTTCTAGATCAAACCTTTATATCCATGACAAC-3'	11
20			

### EXAMPLE 1

#### CLONING OF A HUMAN NR2 (HAEMOPOIETIN RECEPTOR) cDNA

25 A cDNA library constructed from mRNA from a the bone marrow of a patient recovering from chemotherapy was constructed by C. G. Begley, Cancer Research Unit, WEHI in IZAP (Stratagene, CA, USA) were used to infect *Escherichia coli* of the strain LE392. Infected bacteria were grown on twenty 150 mm agar plates, to give approximately 50,000 plaques per plate. Plaques were then transferred to duplicate 150 mm diameter nylon membranes

30 (Colony/Plaque Screen™, NEN Research Products, MA, USA), bacteria were lysed and the DNA was denatured fixed by autoclaving at 100°C for 1 min with dry exhaust. The filters were rinsed twice in 0.1%(w/v) sodium dodecyl sulfate (SDS), 0.1 x SSC (SSC is 150 mM

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sodium chloride, 15 mM sodium citrate dihydrate) at room temperature and pre-hybridised overnight at 42°C in 6 x SSC containing 2 mg/ml bovine serum albumin, 2 mg/ml Ficoll, 2 mg/ml polyvinylpyrrolidone, 100 mM ATP, 10 mg/ml tRNA, 2 mM sodium pyrophosphate, 2 mg/ml salmon sperm DNA, 0.1% SDS and 200 mg/ml sodium azide. The pre-hybridisation buffer was removed. 1.2 mg of the degenerate oligonucleotides for hybridisation (HYB2; Table 2 above) were phosphorylated with T4 polynucleotide kinase using 960 mCi of  $\gamma$ -<sup>32</sup>P-ATP (Bresatec, S.A., Australia). Unincorporated ATP was separated from the labelled oligonucleotide using a pre-packed gel filtration column (NAP-5; Pharmacia, Uppsala, Sweden). Filters were hybridised overnight at 37°C in 80 ml of the prehybridisation buffer containing and  $10^6$  -  $10^7$  cpm/ml of labelled oligonucleotide. Filters were briefly rinsed twice at room temperature in 6 x SSC, 0.1%(v/v) SDS, twice for 30 min at 45°C in a shaking waterbath containing 1.5 l of the same buffer and then briefly in 6 x SSC at room temperature. Filters were then blotted dry and exposed to autoradiographic film at -70°C using intensifying screens, for 7 - 14 days prior to development.

15

Plaques that appeared to hybridise to the probe on duplicate filters were picked and eluted for 2 days at 4°C in 0.5 ml of 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM Tris.HCl pH7.4 containing 0.5%(w/v) gelatin and 0.5% (v/v) chloroform. 5 ml aliquots of each eluate was used as the substrate for two PCR reactions containing 5 ml of 10 x concentrated PCR buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 1 ml of 10 mM dATP, dCTP, dGTP and dTTP, 2.5 ml of the oligonucleotides HYB2 and either T3 or T7 at a concentration of 100 mg/ml, 0.5 ml of Taq polymerase (Boehringer Mannheim GmbH) and water to a final volume of 50 ml. PCR was carried out in a Perkin-Elmer 9600 by heating the reactions to 96°C for 2 min and then for 25 cycles at 96°C for 30 sec, 55°C for 30 sec and 72°C for 2 min. The reactions were then electrophoresed on a 1 %(w/v) low melting point agarose gel in TAE. Any positive products were excised, the gel slice was melted and 2 ml was used as the substrate for a second PCR reaction using conditions identical to the first. The product from the second reaction was purified using an ultrafree-MC centrifugal filtration unit (Millipore Corp.) by centrifugation for 15 min at 2000 g in an eppendorf centrifuge, adding 0.5 ml of 10 mM Tris.HCl, 1 mM EDTA pH8 and recentrifuging. This procedure was repeated three times and the DNA was

30

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recovered in 50 ml of 10 mM Tris.HCl, 1 mM EDTA pH8.

Approximately 500 ng of DNA from each PCR reaction was sequenced using a fmol sequencing kit (Promega Corporation, WI, USA), according to the manufacturer's instructions  
5 with the <sup>33</sup>P-labelled oligonucleotide primer HYB2. The products were resolved on a 6% w/v polyacrylamide gel and the sequence of each clone was analysed using the Blast database comparison programs and the translation function of the Wisconsin suite of DNA programs. The sequence of the PCR product derived from the primary plaque eluate number CF.32  
10 appeared to be novel since it had no homologues in the databases of DNA sequences that were searched, and upon inspection of the sequence of the conceptually translated products appeared also to be a member of the haemopoietin receptor family. This clone was given the name of new receptor 2 or NR2.

The positively hybridising bacteriophage present in the eluate from the primary plug NR2-CF-  
15 32-1 was purified using a second round of screening performed in a manner identical to the first, except that plaques were grown on smaller, 82 mm, plates of agar. Once purified DNA, the positive cDNA cloned into the plasmid pBluescript was excised from the  $\lambda$ -ZAP II bacteriophage according to the manufacturer's instructions (Statagene). A CsCl purified preparation of the DNA was made and this was sequenced on both strands. Sequencing was  
20 performed using an Applied Biosystems automated DNA sequencer, with fluorescent dideoxynucleotide analogues according to the manufacturer's instructions. The DNA sequence was analysed using software supplied by Applied Biosystems.

## EXAMPLE 2

### 25 ISOLATION OF ADDITIONAL NR2 cDNAS

NR2-CF.32 did not appear to contain the entire coding region of the novel receptor. In order to identify other cDNA libraries containing cDNA clones for NR2 we performed PCR upon  
1 ml aliquots of  $\lambda$ -bacteriophage cDNA libraries made from mRNA from various human tissues and using oligonucleotides 722 and 761, designed from NR2-CF-32-1, as primers. The  
30 oligonucleotides are defined in Table 2, above. Reactions contained the same elements as

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described above and were performed in an identical manner. In addition to the original library, five other cDNA libraries appeared to contain NR2 cDNAs. These were screened using a <sup>32</sup>P-labelled oligonucleotide 721 and 761 designed from the 5'-end and the 3' end of the sequence derived from NR2-CF.32, using conditions identical to those described in section (i) except that filters were washed at 55°C rather than 45 °C. Again, as described in section (i), positively hybridising plaques were purified, the cDNAs were recovered and cloned into plasmids pBluescript II or pUC19. Ten independent cDNA clones were sequenced on both strands. Further clones were isolated in a similar manner by screening libraries with oligonucleotide 875 and 939.

10

The extent of each clone is illustrated in Figure 1 and a composite sequence is shown in Figure 2. NR2 clearly has all the features of a member of the haemopoietin receptor family.

### EXAMPLE 3

#### 15 ANALYSIS OF THE EXPRESSION PATTERN OF NR2 mRNA

Northern blots of mRNA from various human tissues and cell lines were hybridised with a random-primed human NR2 cDNA fragment from the internal EcoR I site to the Hpa I site (Figure 1). Using the protocol described previously by Hilton *et al.* (15), two human NR2 mRNA species were observed to be expressed at a low level in a range of adult tissues, and at higher levels in foetal tissues such as the lung and liver. Figure 9 shows expression of NR2 in various mouse tissues using human NR2 cDNA as probe. Interestingly among a series of human haemopoietin cell lines the megakaryocytic cell line MEGO1 expressed high levels of NR2 mRNA suggesting that NR2 and its cognate ligand may play a role in the regulation of the megakaryocyte proliferation, differentiation and/or function.

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**EXAMPLE 4****GENERATION OF PLASMIDS DIRECTING THE EXPRESSION OF  
FULL-LENGTH AND SECRETED FORMS HUMAN NR2**

Since antibodies to NR2 were not available to monitor expression, constructs were engineered  
5 to express full length and two soluble versions of NR2 with an N-terminal "FLAG" epitope  
(International Biotechnologies/ Eastman Kodak, New Haven CT). First, a derivative of the  
mammalian expression vector pEF-BOS was generated so that it contained DNA encoding the  
signal sequence of murine IL-3 (MVLASSTTSIHTMLLLLLMLFHLGLQASIS [SEQ ID NO.  
14]) and the FLAG epitope (DYKDDDDK [SEQ ID NO. 15]) followed by a unique Xba I  
10 cloning site. This vector was named pEF/IL3SIG/FLAG.

The 5' end of the mature NR2 coding region was generated by PCR using primers 1056 and  
721 on clone 60-58-7 (Figure 1). The EcoR I/Hpa I fragment of clone 60-55-7-6 containing  
the 3' end of the NR2 coding region and a portion of the 3'-untranslated region was cloned  
15 into the EcoR I/SmaI digested pBluescript (Figure 1). This construct was digested with Hind  
III and EcoR I and into it was cloned the 5'-NR2 PCR product digested with the same  
enzymes. The resulting construct was digested with Xba I to yield a fragment which contained  
the coding region of human NR2 from Y26 to the natural last amino acid L897 (Figure 1) and  
a segment of 3'-untranslated region and was cloned into the Xba I site of pEF/IL3SIG/FLAG  
20 to give pEF/IL3SIG/FLAG/NR2/897. A soluble derivative of human NR2 was also  
engineered. PCR was carried out either using primers 1056 and 1092 to amplify the predicted  
mature coding region of the extracellular portion of human NR2 (Y26 to D839; Figure 1).  
The PCR products were digested with Xba I and subcloned into Xba I digested  
pEF/IL3SIG/FLAG to give pEF/IL3SIG/FLAG/NR2/839. The identity of each construct was  
25 confirmed by dideoxy sequencing.

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**EXAMPLE 5**  
**TRANSIENT EXPRESSION OF FULL LENGTH AND SECRETED**  
**FORMS OF HUMAN NR2 IN COS CELLS**

In order to confirm that full length and soluble NR2 could be produced using the expression  
5 vectors pEF/IL3SIG/FLAG/NR2/897 and pEF/IL3SIG/FLAG/NR2/839, COS cells were  
transiently transfected with these constructs. Briefly, COS cells from a confluent 175 cm<sup>2</sup>  
tissue culture flask were resuspended in PBS and electroporated (BioRad Gene pulser; 500  
mF, 300 V) with 20 mg of uncut pEF/IL3SIG/FLAG/NR2/897 or  
pEF/IL3SIG/FLAG/NR2/839 in a 0.4 cm cuvette (BioRad). After 2 to 3 days at 37°C in a  
10 fully humidified incubator containing 10% v/v CO<sub>2</sub> in air cells were used for analyses of  
protein expression. Conditioned medium was collected by centrifugation and stored sterile  
at 4°C. Cells were also harvested and lysed for 5 min in 500 µl of 50 mM Tris.HCl pH7.4  
containing 150 mM NaCl, 2 mM EDTA and 1% v/v Triton X-100. The intact nuclei were  
removed by centrifugation at 10,000g for 5 min. 500 µl of 50 mM Tris.HCl pH7.4 containing  
15 150 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100, 1% w/v sodium deoxycholate and 0.2%  
w/v SDS. 15 µl of anti-FLAG M2 affinity gel (International Biotechnologies/ Eastman  
Kodak, New Haven CT) was then added to the cell extract or to 1 ml of conditioned medium  
and precipitation was carried out overnight at 4°C. The affinity gel was then washed three  
times in cold PBS and the precipitated protein was eluted by resuspending the gel in 80 µl of  
20 100 mM sodium phosphate pH7.2, 10 mM EDTA, 0.1% w/v SDS and 1% 2-mercaptoethanol  
and boiling for 5 min. The supernatant was removed and 8 µl of 10% β-octyl glucoside was  
added. One half of each sample was incubated for 16 hours with 0.6 U of N-Glycanase-F  
(Boehringer-Mannheim), while the remainder was left untreated. An equal volume of 2x  
SDS-PAGE sample buffer was added to the samples which were then boiled and  
25 electrophoresed on pre-cast 4-15% w/v polyacrylamide gels (BioRad). The resolved proteins  
were then electroblotted onto Immobolon membranes, which were then blocked with 5% w/v  
skim milk, 0.1% v/v Tween 20 in PBS, rinsed and incubated with 5 µl of anti-FLAG M2  
antibody in 2.5 ml of PBS containing 0.1% v/v Tween 20, rinsed and incubated with  
peroxidase-conjugated human anti-mouse Ig in 5% w/v skim milk, 0.1% v/v Tween 20 in  
30 PBS, rinsed and incubated with ECL reagent for 1 min. Filters were then blotted dry and

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exposed to autoradiographic film for 1 min.

COS cells that were mock transfected contained no reactive protein, while COS cells transfected with pEF/IL3SIG/FLAG/NR2/897 expressed an immunoreactive protein of  
5 between 120,000 and 140,000 molecular weight. Deglycosylation with N-Glycanase-F resulted in a reduction in the apparent molecular weight to approximately 110,000 close to that predicted from the cDNA sequence of NR2. The immunoreactivity observed was completely inhibited by inclusion of an excess of the FLAG peptide during the immunoprecipitation step. No specific immunoreactive proteins could be detected in the medium conditioned by COS  
10 cells transfected with pEF/IL3SIG/FLAG/NR2/897. In contrast immunoreactive proteins were found in the medium and the cell pellet of COS cells transfected with DNA encoding the secreted form of NR2 - pEF/IL3SIG/FLAG/NR2/839. The secreted form of NR2, as predicted, exhibited a lower apparent molecular weight than full length NR2, 110,000 to 120,000. This again decreased upon deglycosylation, to approximately 100,000.

15

COS cells transfected with pEF/IL3SIG/FLAG/NR2/897 were also examined for cell surface expression of NR2 by immunofluorescence staining.  $5 \times 10^5$  COS cells were resuspended in 100 ml of PBS containing 5% fetal calf serum and incubated with FITC-conjugated anti-FLAG M2 antibody for 45 min on ice, the cells were fixed and examined using a fluorescence  
20 microscope. No positive cells were observed in mock transfected samples, while approximately 10% of COS cells transfected with pEF/IL3SIG/FLAG/NR2/897 stained brightly positive. This data was consistent with the expected transient transfection efficiency of COS cells using electroporation.

25

## EXAMPLE 6

### STABLE EXPRESSION OF FULL LENGTH HUMAN NR2

As described below certain routes to the identification of the NR2 ligand require stable expression of full-length NR2 in haemopoietin cell lines and the production and purification of large (mg) amounts of secreted NR2. Stable transfection of the  
30 pEF/IL3SIG/FLAG/NR2/897 and pEF/IL3SIG/FLAG/NR2/839 plasmids was achieved by

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electroporation. Briefly, the plasmids were linearised by digestion with the restriction enzyme Aat II. 20 mg of the linearised pEF/IL3SIG/FLAG/NR2/897 plasmid and 2 mg of pPGKpuropA, pPGKneopA or pPGKhygropA (plasmids directing the expression of the puromycin, neomycin and hygromycin resistance genes) were electroporated into 4 x 10<sup>6</sup> parental Ba/F3 cells, Ba/F3 cells engineered to express human gp130 with or without coexpression of the human LIF receptor, Ba/F3 cells expressing the human b-chain common to the IL-3, IL-5 and GM-CSF receptors, Ba/F3 cells expressing the human IL-2 receptor b- and g-chains, CTLL cells or CHO cells. Briefly, cells were washed twice in ice-cold PBS and resuspended in PBS at 5 x 10<sup>6</sup> per ml. 4 x 10<sup>6</sup> cells were aliquoted into 0.4 mm electroporation 5 curvettes with the DNA. DNA and cells were incubated for 10 min on ice and electroporated at 270 V and 960 mF in a Bio-Rad Gene-Pulser (Bio-Rad Laboratories, CA, USA). The cells were mixed with 1 ml of culture medium, centrifuged through 3 ml of FCS and resuspended in 100 ml of culture medium. Cells were then aliquoted into four 24 well dishes. After two days, selection was commenced by the addition puromycin to a concentration of 20 mg/ml, 15 G418 to a concentration of 1.2 mg/ml or hygromycin to a concentration of 1 mg/ml. After 10 - 14 days, clones of proliferating cells were transferred to flasks and after expansion were tested for receptor expression.

FACS analysis using the anti-FLAG M2 antibody (Figure 3) illustrates that Ba/F3 cells 20 transfected with the pEF/IL3SIG/FLAG/NR2/897 express NR2 on the cell surface. Similar results have been obtained with other cell lines. As with COS cells, CHO cells transfected with pEF/IL3SIG/FLAG/NR2/839 secrete the NR2 extracellular domain. The extracellular domain of NR2 has been purified on an anti-FLAG M2 antibody affinity column using the FLAG peptide as the means of elution. This results in a high degree of purification of the 25 NR2 extracellular domain as seen in the silver-stained poly-acrylamide gel illustrated in Figure 4.

**EXAMPLE 7****STRATEGIES FOR ISOLATION OF THE LIGAND FOR NR2**

The stable expression of full-length and secreted NR2 enables steps to be taken to generate specific monoclonal antibodies to NR2 and allows a number of strategies to be employed to

5 identify the cognate ligands of NR2.

(a) Expression of NR2 in factor dependent cell lines;

A variety of haemopoietin cell lines have been described which are dependent on the presence of exogenous growth factor for survival and proliferation *in vitro*. Among these are the  
10 murine cell lines Ba/F3, FDCP-1, 32D, CTLL, NFS-60, B6SutA, DA-1 and DA-3 and the human cell lines M07 and TF-1. FLAG-tagged murine and human NR2 may be stably expressed in each of these cell lines. The capacity of medium conditioned by a variety of murine and human cell lines and tissues to stimulate the survival and division of factor dependent cell lines expressing NR2 will be compared to the ability of the same medium to  
15 stimulate parental cell lines that do not express NR2. Medium that shows a greater ability to stimulate the proliferation cells expressing NR2 will be considered as a potential source of NR2.

NR2 has also been co-expressed in Ba/F3 cells with the LIF receptor  $\alpha$ -chain and gp130, with  
20 the IL-2 receptor  $\beta$ - and  $\gamma$ -chains of the IL-2 receptor and with the common  $\beta$ -chain of the IL-3, IL-5 and GM-CSF receptors. Again conditioned medium will be tested for their ability to stimulate the proliferation of these cell lines.

(b) Identification of the NR2 ligand using the Cytosensor;

25 The haemopoietin cell lines expressing NR2 described above and additional non-haemopoietin cell lines engineered to express NR2 will be used in conjunction with the Cytosensor to screen conditioned medium for the presence of a ligand capable of altering cellular ion fluxes. Positive conditioned medium will be considered as a potential source of NR2 ligands.

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(c) Selection of Ba/F3 cells expressing the NR2 ligand;

Ba/F3 cells expressing NR2 with or without additional receptor components will be mutated with EMS or with a retrovirus. Mutants that are capable of proliferation in the absence of added growth factor will be selected. The medium from such clones will then be tested for  
5 their ability to stimulate the proliferation of Ba/F3 cells expressing NR2 with or without additional receptor components compared with the corresponding Ba/F3 cells that do not express NR2. Positive conditioned medium will be considered as a potential source of the NR2 ligand.

10 (d) Expression of NR2 in cell lines that may be induced to differentiate;

Similar experiments may be performed by expressing FLAF-tagged NR2 in cells that may be induced to differentiate by cytokines. Such cells include the murine lines M1 and WEHI-3BD+ and the human lines HL-60 and U937. The capacity of medium conditioned by a variety of murine and human cell lines and tissues to induce the differentiation of such cell  
15 lines expressing NR2 will be compared to the ability of the same medium to stimulate parental cell lines that do not express NR2. Medium that shows a greater ability to stimulate the differentiation of cells expressing NR2 will be considered as a potential source of NR2 ligand.

(e) Use of secreted NR2 extracellular domain as a probe on the Biosensor;

20 Purified extracellular domain of NR2 has been obtained and is being immobilized on the surface of a Biosensor chip. Medium conditioned by a variety of murine and human cell lines and tissues will be passed across the chip and specific changes in the surface plasmon resonance will be noted. Positive medium will be considered as a potential source of NR2 ligand.

25

(f) Use of secreted NR2 extracellular domain as the basis of an affinity column;

Purified extracellular domain of NR2 has been obtained and is being immobilized using a variety of chemistries. Affinity columns will be constructed and medium conditioned by a variety of murine and human cell lines and tissues will be passed through. Proteins that bind  
30 to the column will be considered to be candidate NR2 ligands and will be further

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characterised.

### EXAMPLE 8

#### HUMAN LEPTIN

- 5 A human leptin cDNA (16) was cloned into the peFBOS expression vector (17) in frame with the interleukin-3 leader sequence followed by the FLAG<sup>TM</sup> epitope sequence (18). CHO cells were transfected with this vector by electroporation and supernatant harvested from exponentially growing cultures. The supernatant was concentrated over a YM-10 membrane (10-fold) and then applied to an affinity column containing immobilised anti-FLAG<sup>TM</sup>
- 10 antibody M2. The column was eluted with FLAG<sup>TM</sup> peptide according to the manufacturer's instructions (Eastman Kodak, Rochester, NY). The monomeric form of human leptin was purified by gel filtration chromatography on a Superose 12 column (Pharmacia, Uppsala, Sweden) and exchanged into 20 mM phosphate buffered (pH7.4) saline (0.15 M) containing 0.02% v/v Tween 20 and 0.02% w/v sodium azide (PBS) by gel filtration on Sephadex G-25
- 15 M (PD-10) columns (Pharmacia). Human leptin was iodinated with <sup>125</sup>I using a modified iodine monochloride method (19) to a specific radioactivity of approximately 10<sup>7</sup> cpm/pmol and exchanged into PBS as above.

### EXAMPLE 9

#### 20 BINDING OF <sup>125</sup>I HUMAN LEPTIN TO CELLS EXPRESSING NR2 OR TO SOLUBLE NR2

Cos-hNR2 are COS-7 cells electroporated with peFBOX-hNR2 and harvested at 3½ days (5x10<sup>4</sup> cells used per point).

- 25 Ba/F3-hNR2 are Ba/F3 cells stably transfected with peFBOS-hNR2 (9x10<sup>5</sup> cells used per point).

Solh NR2 is a soluble form of human NR2 purified by anti-FLAG<sup>TM</sup> affinity chromatography from the supernatant (48 hr) of COS cells transfected with peFBOS-solh NR2 (approx. 0.1

30 µg/ml final concentration in binding assay).

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For cells, the total reaction volume was 100  $\mu$ l in RPMI-medium containing 10 mM Hepes pH7.4 and 10% v/v foetal calf serum (RHF). The reaction mixture also contained  $^{125}$ I h leptin 0-6x10<sup>5</sup> cpm as indicated with or without unlabelled h leptin (approx. 1  $\mu$ g/ml).

5 The mixture was incubated for 1-1.5 hr at 23°C and then layered over 200  $\mu$ l cold foetal calf serum in small, tapered centrifuge tubes (Elkay, Melbourne) and centrifuged at 12000 g for 10 sec. The cell pellet was removed by cutting the tubes with a scalpel blade and the cell bound (pellet) radioactivity and the unbound radioactivity (the rest of the tube) were separately counted in a Packard  $\gamma$ -counter. Specifically bound  $^{125}$ I h leptin was determined as the  
10 difference in counts between otherwise identical tubes that contained or did not contain the unlabelled excess h leptin. The data were plotted as saturation curves (specifically bound versus added  $^{125}$ I h leptin) and as Scatchard transformations (specific bound/free radioactivity versus specific bound radioactivity to determine the equilibrium dissociation constants [ $K_d$ ]  
(20).

15

For soluble receptors (sol hNR2) incubations were as above but after 1 hr at 23°C, 20  $\mu$ l of concavalin A-sepharose 4B beads (¼ suspension in 0.1 M acetate pH5) were added and incubation continued for a further 30 min. Subsequently, the beads were centrifuged and processed as above. The results are shown in Figures 5 and 6. Human leptin binds to  
20 Ba/F3/COS cells transfected with hNR2 cDNA and to soluble hNR2.

## EXAMPLE 9

### EXPRESSION OF NR2 IN ANIMAL SPECIES

Genomic DNA from various sources was digested with EcoRI. This was then blotted onto a  
25 nylon membrane (GeneScreen Plus®, NEN Research Products, USA). The filter was then probed using a 1.1 kb cDNA fragment of NR2. The fragment covers the 3' half of the first haemopoietin domain and extends to cover the whole of the second haemopoietin domain, terminating the type III fibronectin domain. The filter was prehybridised and hybridised in 0.5M sodium phosphate, 7% w/v SDS and 1mM EDTA at 50°C overnight. The filter was  
30 then washed in 40 mM sodium phosphate and 1% w/v SDS at 50°C. The results are shown



in Figure 7.

#### EXAMPLE 10

##### CLONING OF THE HUMAN NR2 LOCUS

- 5 In order to obtain genomic clones of the human NR2 locus, various genomic libraries were screened. These libraries were screened with either oligonucleotide or cDNA probes. Oligonucleotide screening conditions:  $1 \times 10^6$  clones were fixed to nylon filters (Colony/Plaque Screen<sup>TM</sup>, NEN Research Products, USA). These filters were then prehybridised in a 6xSSC buffer containing 0.2% Ficoll, 0.2% w/v bovine serum albumin, 0.2% polyvinylpyrrolidone,
- 10 0.1M ATP, 50  $\mu$ g/mL transfer RNA, 2 mM tetra-sodium pyrophosphate, 50  $\mu$ g/mL herring sperm DNA and 0.1% w/v sodium azide at 37°C for at least 2 hours. They were hybridised overnight under the same conditions, with at least  $2 \times 10^6$  cpm/mL of P-labelled oligonucleotide probe. The filters were then washed in 6x SSC/0.1% w/v SDS at 50-55°C depending on the sequence of the specific oligonucleotide (Melting Temp -10°C).
- 15 cDNA screening conditions:  $1 \times 10^6$  clones were fixed to nylon filters. These filters were then prehybridised in a 2xSSC buffer containing 0.2% Ficoll, 0.2% w/v bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.1M ATP, 50  $\mu$ g/mL transfer RNA, 2mM tetra-sodium pyrophosphate, 50  $\mu$ g/mL herring sperm DNA and 0.1% w/v sodium azide at 37°C for at least
- 20 2 hours at 65°C. They were hybridised overnight under the same conditions, with at least  $2 \times 10^6$  cpm/mL of <sup>32</sup>P-labelled cDNA fragment. The filters were then washed in 2xSSC/0.1% w/v SDS at 65°C.

#### EXAMPLE 11

##### RESTRICTION ENZYME MAPPING

- 25 The clones obtained were characterised by mapping with partial endonuclease digestion (21).
- In order to determine on which fragments the various exons were present, specific oligonucleotide probes were used. The various clones were digested with a range of restriction
- 30 enzymes. These were then blotted to a nylon membrane (GeneScreen Plus®), NEN Research

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Products, USA). Oligonucleotides derived from the cDNA sequence (and known to be specific for a particular exon), were then hybridised to the digested fragments. These hybridisations were done under the same conditions as mentioned above for oligonucleotides. Exons could then be mapped to specific fragments by a positive hybridisation after overnight  
 5 exposure.

Intron/exon boundary sequences were determined by sequencing across the intron/exon boundaries. Primers specific for sequence on either side of the boundary were used in a sequencing PCR reaction. Sequencing was performed on an ABI 373 sequencer using the Taq  
 10 cycle sequencing kit (Applied Biosystems). These sequences were then compared to the consensus intron/exon boundary sequence (22). The results are shown in Figure 8 and in Table 3.

#### EXAMPLE 12

##### 15 DETERMINATION OF AMINO ACID SEQUENCE OF hNR2

The N-terminal amino acid sequence of hNR2 was determined. The results are shown below. The actual sequence starts at amino acid 16. The sequence is as follows:

20	Asp Ser Ile Ser Ser Ser Asp Tyr Lys Asp Asp Asp Glu Ser Arg	15
	5 10	
	Tyr Pro Ile Thr Pro Trp Arg Phe Lys Leu Ser Xaa Met Pro Pro	30
	20 25	
25	Xaa Ser Thr Tyr Asp [SEQ ID NO:31]	
	35	

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that  
 30 the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

## CLAIMS:

1. An isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a haemopoietin receptor or a derivative thereof wherein said sequence of nucleotides or a complementary form thereof is capable of hybridising under medium stringent conditions to the oligonucleotide:

5'-(A/G)CTCCA(A/G)TC(A/G)CTCCA-3' [SEQ ID NO:1].

2. An isolated nucleic acid molecule according to claim 1 wherein said nucleic acid molecule comprises a nucleotide sequence or a complementary form thereof which hybridises under medium stringent conditions to the oligonucleotides:

5'-ACTAGCAGGGATGTAGCTGAG-3' [SEQ ID NO:4]

5'-CTGCTCCTATGATACCT-3' [SEQ ID NO:6]

5'-CCTCTTCCATCTTATTGCTTGG-3' [SEQ ID NO:7]

5'-ATCGGTCGTGACATAACAAG-3' [SEQ ID NO:8].

3. An isolated nucleic acid molecule according to claim 2 wherein said nucleic acid molecule comprises a nucleotide sequence or a complementary form thereof which hybridises under medium stringent conditions to one or more of the following oligonucleotides:

5'-CTCAGCTACATCCCTGCTAGT-3' [SEQ ID NO:5]

5'-AGCTAAGCTTTCTAGATATCCAATTACTCCTTGGAGA-3' [SEQ ID NO:9]

5'-AGCTTCTAGATCAATCACTCTGGTGTTTTTCAAT-3' [SEQ ID NO:10]

5'-AGCTTCTAGATCAAACCTTTATATCCATGACAAC-3' [SEQ ID NO:11].

4. An isolated nucleic acid molecule according to claim 3 wherein the haemopoietin receptor is capable of interaction with leptin.

5. An isolated nucleic acid molecule according to claim 4 comprising a nucleotide sequence as set forth in SEQ ID NO:12 or is capable of hybridising to all or part thereof under low stringent conditions.

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6. A recombinant haemopoietin receptor or a derivative thereof encoded by a nucleic acid molecule which comprises a nucleotide sequence or a complementary form thereof which is capable of hybridising to SEQ ID NO:1 under medium stringent conditions.

7. A recombinant haemopoietin receptor or its derivative according to claim 6 wherein said haemopoietin receptor is encoded by a nucleic acid molecule which comprises a nucleotide sequence or a complementary form thereof which is capable of hybridising to SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8 under medium stringent conditions.

8. A recombinant haemopoietin receptor or its derivative according to claim 7 wherein said haemopoietic receptor is encoded by a nucleic acid molecule which comprises a nucleotide sequence or complementary form thereof which hybridises under medium stringency conditions to one or more of SEQ ID NO:1 and SEQ ID NO:4 to SEQ ID NO:11.

9. A recombinant haemopoietin receptor or its derivative according to claim 8 wherein the haemopoietin receptor is capable of interaction with leptin.

10. A recombinant haemopoietin receptor or its derivative according to claim 9 encoded by a nucleic acid molecule comprising a nucleotide sequence or complementary form thereof substantially as set forth in SEQ ID NO:12 or a sequence capable of hybridising to all or part thereof under medium stringent conditions.

11. A recombinant haemopoietin receptor or its derivative according to claim 10 wherein said haemopoietin receptor has an amino acid sequence substantially as set forth in Figure 2 [SEQ ID NO:13] or having at least about 60% similarity to all or part thereof.

12. A nucleic acid molecule according to claim 1 or claim 6 wherein said haemopoietin receptor is of mammalian origin.

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13. A nucleic acid molecule according to claim 12 wherein the haemopoietin receptor is derived from a human, livestock animal, laboratory test animal, companion animal or captive wild animal.
14. A nucleic acid molecule according to claim 13 wherein the haemopoietin receptor is derived from a human or murine species.
15. An antibody to the recombinant haemopoietin receptor according to any one of claims 6 to 11.
16. An antibody according to claim 15 wherein the antibody is a monoclonal antibody.
17. A ligand capable of binding to a haemopoietic receptor according to any one of claims 6 to 11.
18. A ligand according to claim 17 wherein the ligand is leptin.
19. A method of identifying a ligand capable of interacting with a haemopoietic receptor as defined in any one of claims 6 to 11, said method comprising contacting a biological sample containing a putative ligand with said haemopoietic receptor or a ligand binding portion thereof immobilised to a solid support for a time and under conditions sufficient for a complex to form between said receptor and said ligand if said ligand is present in said biological sample, eluting bound ligand and isolating same.
20. A pharmaceutical composition comprising a recombinant haemopoietin receptor according to any one of claims 6 to 11 or a ligand binding portion thereof and one or more pharmaceutically acceptable carriers and/or diluents.

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21. A pharmaceutical composition comprising a ligand to the recombinant haemopoietin receptor according to any one of claims 6 to 11 and one or more pharmaceutically acceptable carriers and/or diluents.

22. A method of treatment in a mammal comprising administering to said mammal a treatment effective amount of a recombinant haemopoietin receptor according to any one of claims 6 to 11 or a ligand binding portion thereof or a ligand to said haemopoietic receptor for a time and under conditions sufficient for said treatment to be substantially effected or substantially ameliorated.

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 96/00607

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
Int Cl <sup>6</sup> : C12N 15/11, 15/12; C07K 16/28; A61K 38/17; G01N 33/566		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols) C12N, C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CHEM ABS via STN: [AG] CTCCA [AG] TC [AG] CTCCA OR TGGAG [TC] GA [TC] TGGAG [TC] AND (HAEMOPOIETIN OR HEMOPOIETIN)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AU, A, 34194/95 (PROGENITOR, INC.) 21 March 1996 Claims and Fig. 2	1-22
P, X	Cell, Vol. 83 (7), 29 December 1995, TARTAGLIA, L.A. et al, "Identification and Expression Cloning of a Leptin Receptor, OB-R", pages 1263-1271 Figs. 3 and 4	1-22
P, X	Cell, Vol. 84 (3), 9 February 1996, CHEN, H. et al, "Evidence that the Diabetes Gene Encodes the Leptin Receptor: Identification of a Mutation in the Leptin receptor Gene in db/db Mice", pages 491-495 Page 494 and Fig. 1	1-22
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 21 November 1996		Date of mailing of the international search report 04.12.96
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929		Authorized officer  BARRY SPENCER Telephone No.: (06) 283 2284

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 96/00607

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages (Remove spaces when completed if the page is too long)	Relevant to claim No.
P,X	Nature, Vol. 379 (6566), 15 February 1996, GWO-HWA LEE et al, "Abnormal splicing at the Leptin Receptor in Diabetic Mice", pages 632-635 Whole document	1-22
P,X	Proc. Natl. Acad. Sci. USA, Vol. 93 (13), June 1996, GHILARDI, N. et al, "Defective STAT Signalling by the Leptin Receptor in Diabetic Mice", pages 6231-6235 Page 6232	1-22
P,X	Biochem. Biophys. Res. Comm., Vol. 222 (1), 1996, IIDA, M. et al, "Phenotype-Linked Amino Acid Alteration in Leptin Receptor cDNA from Zucker Fatty (fa/fa) Rat", pages 19-26 Figure 2	1-22
P,X	Nature Medicine, Vol. 2 (5), May 1996, CIOFFI, J.A. et al, "Novel B219/OB Receptor Isoforms: Possible Role of Leptin in Hematopoiesis and Reproduction", pages 585-589 Fig. 1	1-22
P,X	Nature Genetics, Vol. 13 (1), May 1996, PHILLIPS, M.S. et al, "Leptin Receptor Missense Mutation in the Fatty Zucker Rat", pages 18-19 Fig. 2	1-22
P,X	Biochem. Biophys. Res. Comm., Vol. 224 (2), 1996, IIDA, M. Et al, "Substitution at codon 269 (Glutamine → Proline) of the Leptin Receptor (OB-R) cDNA is the only Mutation Found in the Zucker Fatty (fa/fa) Rat", pages 597-604 Fig. 1	1-22



### Information on patent family members

**PCT/AU 96/00607**

Patent Document Cited in Search Report		Patent Family Member					
AU	34194/95	CA	2176463	EP	730606	WO	9608510

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